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Reduction of a 4q35-Encoded Nuclear Envelope Protein in Muscle Differentiation

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Abstract

Muscular dystrophy and peripheral neuropathy have been linked to mutations in genes encoding nuclear envelope proteins; however, the molecular mechanisms underlying these disorders remain unresolved. Nuclear envelope protein p19A is a protein of unknown function encoded by a gene at chromosome 4q35. p19A levels are significantly reduced in human muscle as cells differentiate from myoblasts to myotubes; however, its levels are not similarly reduced in all differentiation systems tested. Because 4q35 has been linked to facioscapulohumeral muscular dystrophy (FSHD) and some adjacent genes are reportedly misregulated in the disorder, levels of p19A were analyzed in muscle samples from patients with FSHD. Although p19A was increased in most cases, an absolute correlation was not observed. Nonetheless, p19A downregulation in normal muscle differentiation suggests that in the cases where its gene is inappropriately reactivated it could affect muscle differentiation and contribute to disease pathology.

Keywords

nuclear envelope; muscle differentiation; facioscapulohumeral muscular dystrophy; laminopathy

Introduction

The nuclear envelope (NE) is a complex subdomain of the nucleus that is linked to multiple diseases including those affecting striated muscle. Mutations in *LMNA*, which encodes A-type lamins, cause several disorders characterized by dilated cardiomyopathy with variable skeletal muscle involvement, including Emery-Dreifuss muscular dystrophy (EDMD) and limb-girdle

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muscular dystrophy type 1B (LGMD1B) [1;2;3;4]. Mutations in *EMD* encoding emerin cause X-linked EDMD [5]. The molecular mechanisms by which mutations in genes encoding NE proteins produce pathology in striated muscle remain unclear, but potential causes that are supported experimentally include mechanical instability of the nucleus, altered gene expression, disruption of cell signaling cascades, and failure of satellite cell function [4;6]. This wide range of affected functions suggests that additional interacting NE proteins are involved in developing disease pathologies and implies that such partner proteins could be involved in additional muscle disorders.

Partner proteins could be among 67 previously uncharacterized nuclear membrane proteins identified by proteomics [7]. The human homolog of one of these proteins, p19A (initially called NET19), is encoded in the q35 region of chromosome 4 that is linked to fascioscapulohumeral muscular dystrophy (FSHD), one of the most common hereditary myopathies that has some clinical features similar to the NE-linked limb-girdle muscular dystrophy (type 1B), beginning with facial muscle degeneration but progressing to limb-girdle muscles and deltoids [3;8]. FSHD is not associated with point mutations in any specific gene: rather, over >90% of cases correlate with a reduced number of D4Z4 repeats at 4q35. Wild-type alleles of some 4q35 genes are upregulated in the disease, though the molecular mechanism remains unresolved [9;10;11]. As *p19A* is located in 4q35 we considered that its misregulation might contribute to FSHD pathology. p19A levels were higher in some patients, but a clear correlation could not be made due to high variation among both control and patient samples. Strikingly, p19A protein levels are greatly reduced during muscle differentiation and absent from or expressed at low levels in adult muscle. Thus in those patients in which it is upregulated, p19A could contribute to muscle pathology.

Materials and methods

Original Predicted NET19 Exons

Amino acid sequences predicted from each exon of the original orf XP_298567.

1:MKLELRVPVVLVDVFTEMATLLTIITRLVKNWVDLWPRQENG

2:FILVSSWLTLFLSPGTLVAMFCDTKWKTENIDLQLERRMEQR

3:CSMFLAVESTRTSAVVQEKPAQGGLCVRTAQWAVWSRFALSVRPELARTCRKLL
RSSGGA

4:GHLRGLASFRSRIELRRQIARPHAAGRETCCPLRRNHAGAPRLPSACSPPEVPVSR
VLMRGLKRKRGRRRARDPKGRGGEEETLALSFSCQWQPPPQ

Antibody Generation

Peptides (3.5 mg each) representing four regions in the XP_298567 coding sequence (a = TRLVKNWVDLWPRQENG, b = DTKWKTENIDLQLERRME, c = NHAGAPRLPSACSPPEVP, and d = LKRKRGRRRARDPKGRGGE) were conjugated to keyhole limpet hemocyanin (KLH; 7 mg) with sulfo-SMCC (Pierce Chemical Co.; 1 mg). Free crosslinker was removed on a PD10 column (Pharmacia) and 500 µg of complexed peptide was emulsified with TiterMax Classic Adjuvant (Sigma) for injection 1 and Freund's incomplete adjuvant (Sigma) for subsequent injections (3-week intervals) into New Zealand White rabbits. Actin antibodies (A-1978 Sigma) and lamin A (2962) and B1 (3931) antibodies [12] were previously described.

Cell Culture and Differentiation

All non-differentiation media contained 10% fetal calf serum, 100 µg/ml penicillin and 100 µg/ml streptomycin sulfate (Invitrogen). This was added to high glucose DMEM (Invitrogen) for HT1080, HeLa, COS-7, 293T and C2C12 cells, DMEM with MEM non-essential amino acids (Gibco) and 1 mM Na-Pyruvate (Gibco) for 3T3-L1 cells, RPMI (Gibco) for HL-60 and EL-4 cells, and SKGM (Cambrex, CC-3160) for primary myoblasts.

Myoblasts were isolated from patient and control samples using approved protocols and ethics considerations. Cultures were split and half was cultured in 2% horse serum medium for 6–9 days to differentiate into myotubes. Confluent C2C12 cells were differentiated in DMEM containing 0.1 % FCS, Na-pyruvate, 5 µg/ml insulin and 5 µg/ml transferrin. Two days post-confluent 3T3-L1 cells were differentiated with medium containing 1 µM dexamethasone, 0.5 mM 3-isobutyl-1-methylxanthine (IBMX), and 10 µg/ml insulin that was replaced after 2 days. At 4 days fresh medium containing just insulin was added. HL-60 cells (ATCC) were differentiated like macrophages with 10 nM TPA (phorbol 13 myristate acetate; Calbiochem) or like granulocytes with 1 µM *trans*-retinoic acid (Sigma).

For cell doubling measurements daily averages of at least 5 haemocytometer counts for suspension cultures and 20 grids on CELLocate coverslips (Eppendorf) were used to plot the number of cell divisions per unit time.

Subcellular Fractionation

Rat liver NEs and microsomes were isolated as previously detailed [13]. For salt extractions, 6×10^7 NEs were resuspended in 500 mM NaCl, 25 mM Tris pH 8.0, incubated on ice 15 min, and then centrifuged at $10,000 \times g$ for 30 min to separate supernatant and pellet fractions. For alkali extractions, the NEs or microsomes were instead resuspended in 0.1 N NaOH, 0.01 M DTT and immediately centrifuged at $50,000 \times g$ for 30 min. An equivalent percentage of supernatant and pellet were loaded onto gels.

Western Blotting

PVDF membranes were blocked in 10% milk, incubated with primary antibodies for 1 h, washed with 0.2% Tween-20, and incubated with anti-rabbit IgG-HRP 25 min followed by further washes. Membranes were then incubated in ECL (Pierce) 5 min and exposed to Biomax Light autoradiography film (Kodak).

Results and discussion

A protein initially termed NET19 was identified by tandem mass spectrometry of a complex peptide mixture generated from a NE fraction [7]. Fragmentation spectra of the peptide yielded a high confidence match to the translated sequence (XP_298567) of hypothetical orf (XM_298567) located at 4q35 in the human genome encoding a 242 amino acid protein. This database entry was subsequently removed as several different contig assemblies rendered the precise sequence uncertain, presumably related to its proximity to the D4Z4 repeat sequences at 4q35. To test the validity of the original mass spectrometry identification four peptide antibodies were generated based on three of the four exons of the original annotated sequence that spanned roughly 40 Kb (Fig. 1A, B), all of which remained in the genomic sequence for 4q35 (AC093909, Homo sapiens BAC clone RP11-756P1). Antibodies to peptides from exons 1 and 2, 30 kb distant from one another, both recognized a 70 kDa band on Western blots of HeLa cells and rat liver NEs (Fig. 1C, left). In contrast, both antibodies to exon 4 peptides recognized a prominent 50 kDa protein in HeLa lysates, which was not clearly evident in rat liver NEs (Fig. 1C, right). Thus, the original predicted exons 1 and 2 encode parts of a 70 kDa protein, which we refer to as p19A, although they only account for 14% of its mass. Several

attempts to amplify a fragment of p19A cDNA by PCR from a human liver cDNA library using various hypothetical exon combinations based on splice junction prediction algorithms were unsuccessful, so the complete sequence remains unknown. The original predicted exon 4 encodes part of a second 50 kDa protein, which we refer to as p19B.

The two proteins had very distinctive properties when tested for subcellular compartmentalization and membrane insertion. The p19A antibodies reacted with a NE fraction, but did not react with a microsome fraction on Western blots (Fig. 2A). In contrast the p19B antibodies reacted with a protein in the microsomes and failed to react with NEs. Thus, only p19A can be classified as a NE protein. Each confirmed p19A exon has a strongly predicted transmembrane segment by TMHMM version 2.0 [14]. To further characterize p19A membrane association, NEs were extracted with either 500 mM NaCl or 0.1 N NaOH and probed by Western blot with antibodies. LAP2 β served as control, being a well-characterized integral nuclear membrane protein. Both p19A and LAP2 β strongly resisted extraction with 500 mM salt; however almost no LAP2 β was released from NEs with the alkali treatment, while most of the p19A was solubilized (Fig. 2B). The weaker membrane association is not inconsistent with membrane insertion as other NE transmembrane proteins have quite varied extraction properties [15;16], but p19A might also be inserted as a “monotypic” protein that does not span the two bilayers.

As p19A and p19B are located in a chromosomal region linked to FSHD, we tested if, like some other genes at 4q35 [9;10;11], they are upregulated in FSHD patients. Myoblasts were isolated from four patient samples and controls that were coded so that analysis would be unbiased. All patients had <28 kb EcoRI fragments of the D4Z4 repeats, consistent with FSHD linked to reduced repeat number. Equivalent sample loading was based on Coomassie blue staining and confirmed by staining for actin and lamins. The p19A level was higher in three of the four patient samples than in all four controls and much higher than three controls (Fig. 3A). The p19A level in the fourth patient sample was still stronger than half of the controls. Thus, though upregulation of p19A could not be absolutely correlated with FSHD, correlation could be masked by the variable expression in both disease and control samples.

To understand the extent of this variability, protein lysates from adult human muscle of FSHD patients and controls were probed for p19A. No p19A band was detectable in any of these samples, though it was readily detectable in HeLa cell lysate loaded on the same gel (Fig. 3B, left). Furthermore, lamin protein was readily detectable in the muscle samples (Fig. 3B, right); so p19A should have been detectable if present. The absence of p19A in adult muscle and variable levels observed in isolated myoblasts suggested that p19A levels might be reduced in muscle differentiation. To test this hypothesis, myoblasts isolated from patient samples and controls were induced to differentiate into myotubes by serum withdrawal, similar amounts of total protein were run on gels, and p19A was detected by immunoblotting (Fig. 3C). In every instance, regardless of the variable starting levels, the levels of p19A dropped precipitously upon differentiation. In contrast p19B levels were essentially unchanged.

One possible explanation for the variability in basal levels of p19A in myoblasts was suggested by the observation that those with lower levels took much longer to double than those with higher levels. To test if higher p19A protein levels correlate to a faster cell doubling time, protein lysates from a variety of cell lines with different doubling times were compared for levels of p19A, p19B, and lamin B1 (Fig. 4A). However, in contrast to primary myoblasts, immortalized cell lines with shorter doubling times did not have higher levels of p19A. 293T and EL-4 cells, which had respective doubling times of 11 and 14 h, had very low p19A levels. In contrast, HT1080, HeLa, COS7, and 3T3-L1 cells which all doubled at >20 h expressed high levels of p19A (Fig. 4A, B). Levels were also high in C2C12 cells (doubling time, 19 h). In contrast, levels of p19B were similar in all lines except the suspension EL-4 cells.

To test if p19A loss is a general characteristic of cellular differentiation, p19A levels were also measured in four *in vitro* differentiation systems: muscle (C2C12), adipocyte (3T3-L1), macrophage-like (HL-60), and granulocyte-like (HL-60). Levels of p19A dropped below the level of detection during C2C12 differentiation and to very low levels during 3T3-L1 differentiation (Fig. 4B). As serum is not reduced in 3T3-L1 differentiation, the loss of p19A is not related to serum withdrawal employed to differentiate myoblasts to myotubes. Another variable is introduced with HL-60 cells, which in contrast to the other lines are grown in suspension. When treated with retinoic acid, they withdraw from the cell cycle and differentiate along a granulocytic pathway while remaining in suspension [17]. When treated with phorbol esters, they adhere to a substratum and spread as they acquire macrophage-like characteristics [17]. The levels of p19A were maintained during the granulocytic differentiation (Fig. 4C, dg), while they were dramatically reduced during the macrophage-like differentiation (Fig. 4C, dm). As cells in both differentiation paradigms withdraw from the cell cycle, the loss of p19A in just the macrophage-like differentiation appears to be unrelated to withdrawal from the cell cycle. The only shared characteristic of all differentiation systems in which p19A was lost is that the cells underwent significant morphological changes (myoblast fusion, fat deposition, substratum adherence and spreading).

This suggests that p19A may be involved in a nuclear function that must be removed to enable tissue remodeling. The morphological changes in these differentiation systems would require repositioning of nuclei and restructuring of nucleo-cytoskeletal connections as overall cell shape changes. As p19A was most completely lost in muscle differentiation it is tempting to speculate that failure to remove p19A might prevent satellite cells from differentiating to replace damaged muscle. Such an effect could contribute to the pathophysiology of FSHD in the patients who exhibited upregulation of p19A.

The mechanism underlying the pathophysiology of FSHD remains contentious, but it is generally agreed that loss of D4Z4 repeats and consequent changes in the epigenetic makeup of the surrounding region are significant factors, likely affecting the expression of multiple genes [18]. Until the complete p19A gene can be cloned it will not be possible to form a detailed molecular model for how it might contribute to FSHD or other disorders. However, it is intriguing that this is the first 4q35-encoded protein in NEs. A potential link between FSHD and the NE came from observations that the 4q telomere occurs preferentially at the nuclear periphery [19] and is associated with peripheral heterochromatin [20]. This finding is interesting in relation to the idea of a position effect in FSHD; however, the position effect might relate to proximity to the epigenetically silent environment of the NE [21;22] if a threshold number of D4Z4 repeats is required for NE tethering. NE tethering of 4q35 might also explain why only reduction in D4Z4 repeats at 4q35 causes FSHD while reduction in other D4Z4 repeats at 10q26 has no disease correlation [23].

The considerable clinical variability of both FSHD and the laminopathies LGMD1B and EDMD is consistent with the high degree of variability in expression levels for p19A observed here. Thus whatever the precise role of p19A in cellular/tissue remodeling and 4q tethering to the periphery, p19A is a reasonable candidate to influence the pathology of FSHD or muscle laminopathies if inappropriately expressed in adult cells.

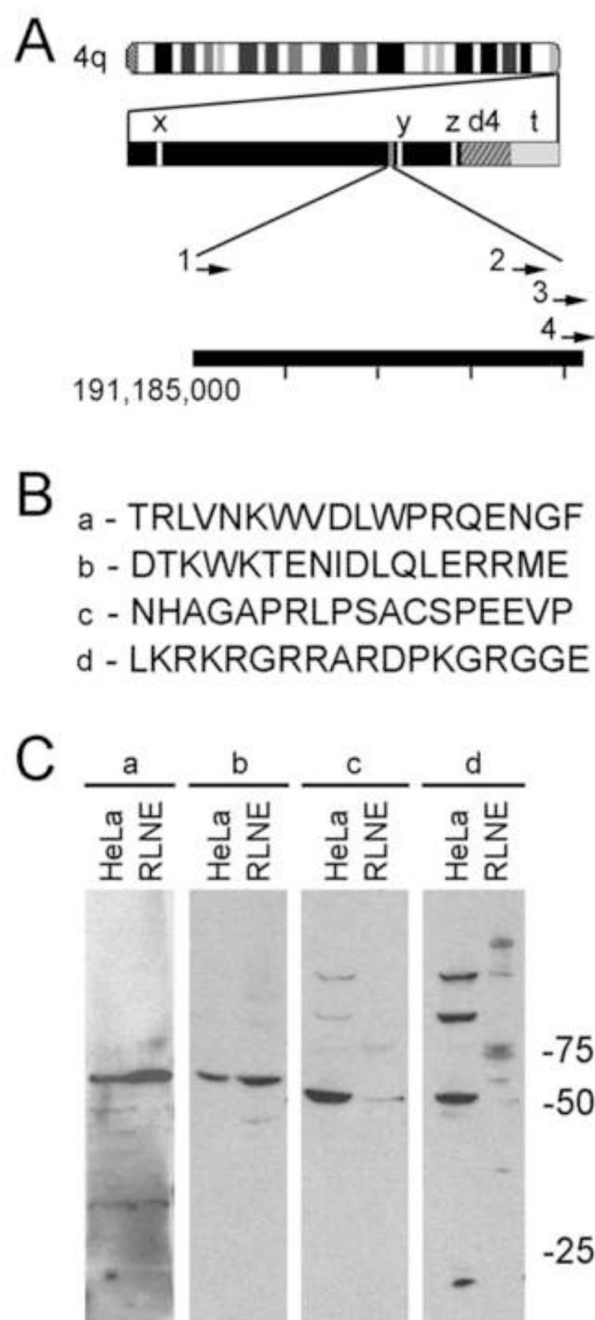
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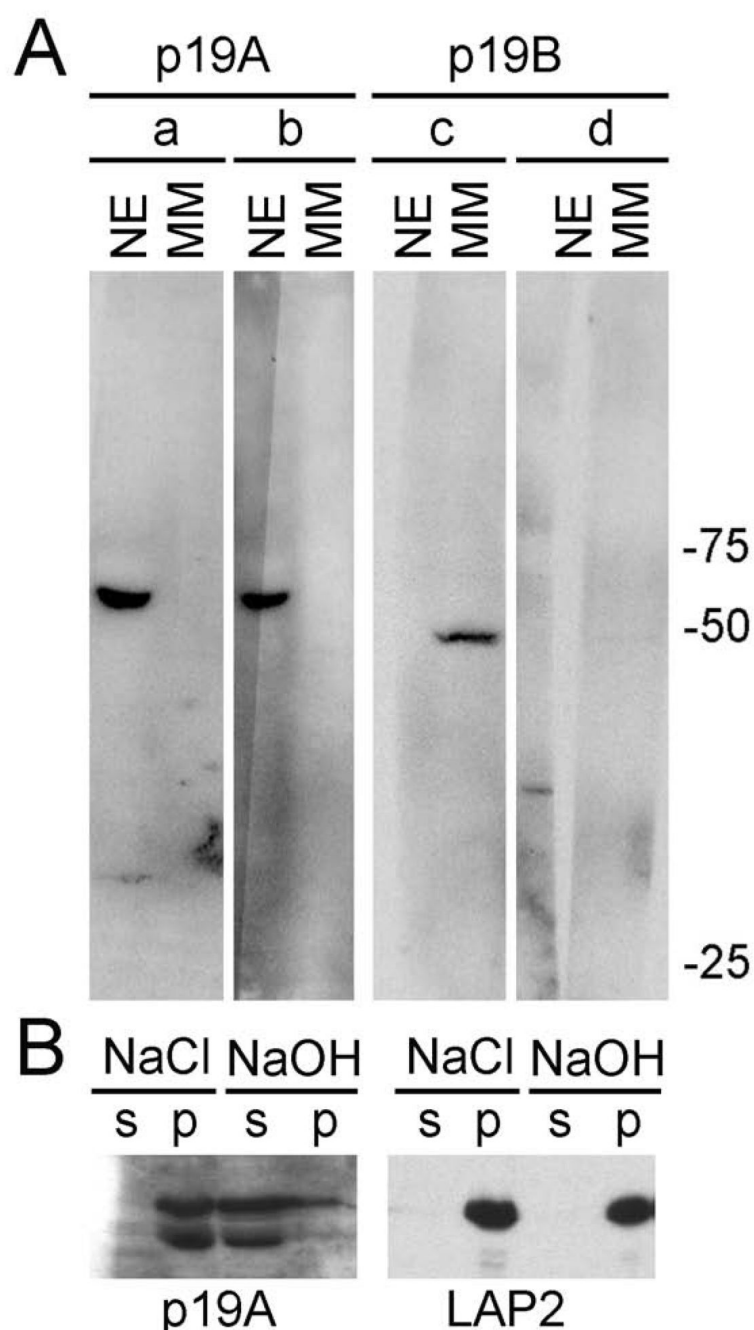
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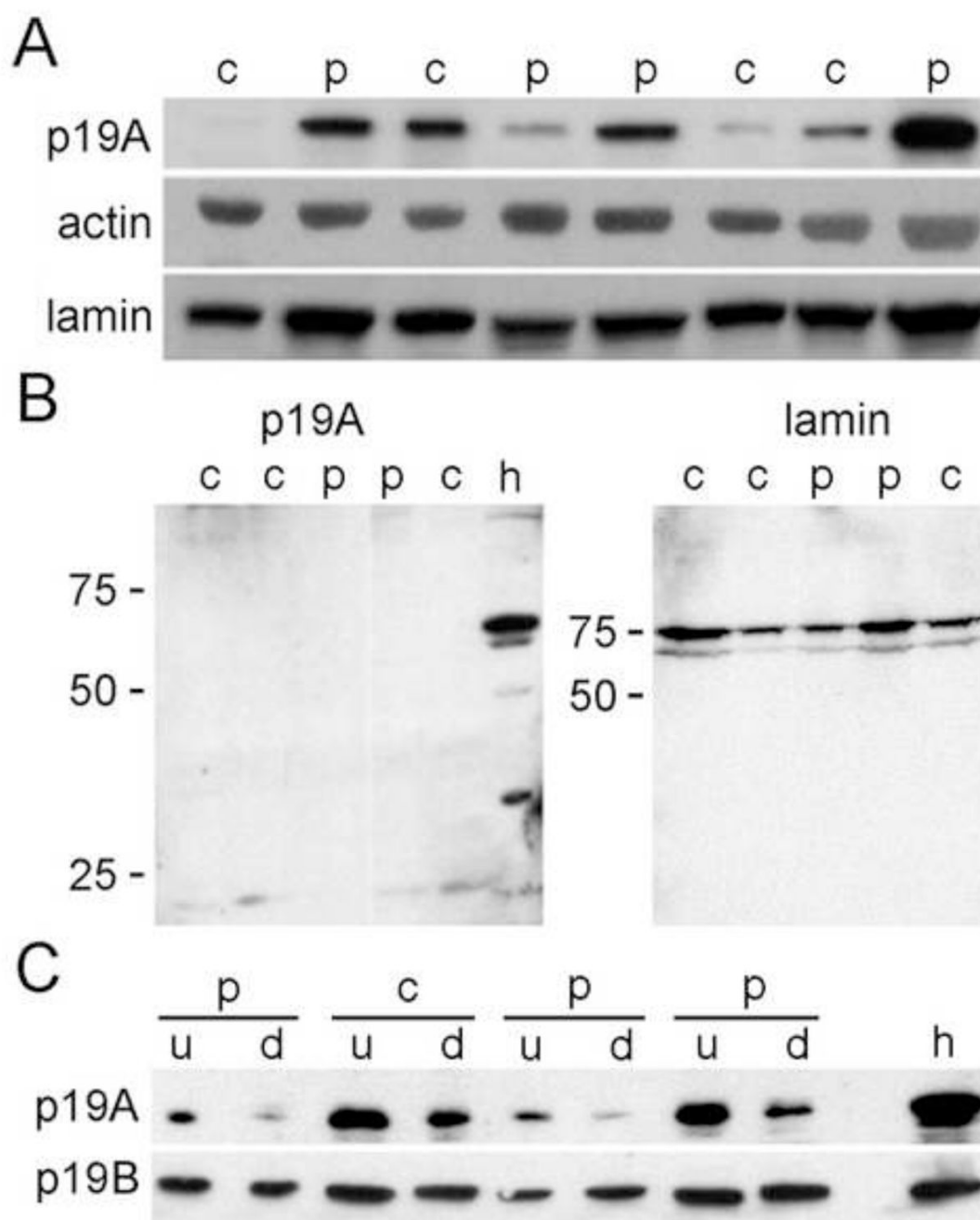
**Fig. 1.**

The original NET19 hypothetical orf encompasses two different proteins. (A) NET19 genomic structure. 4q35 expansion showing scaled locations of several genes/motifs suggested to be involved in FSHD: ANT1 (x), FRG1 (y), FRG2 (z), D4Z4 repeats (d4), telomere (t), and position of NET19, 273 kb upstream of FRG1 and 2 Mb upstream of the D4Z4 repeats. Further expansion of NET19 exons (crossbars, 10 kb). Exon 1 starts at 191,185,000 on chromosome 4 and roughly 30 kb spans between predicted exons 1 and 2. (B) Peptides used to generate antibodies: a in exon 1, b in exon 2, and c and d in exon 4. (C) These antibodies were used to detect proteins in HeLa cells and rat liver NEs (RLNE). Antibodies to peptides a and b both

recognize the same 65–70 kDa band subsequently referred to as p19A, while antibodies c and d both recognize a distinct ~50 kDa protein subsequently referred to as p19B.

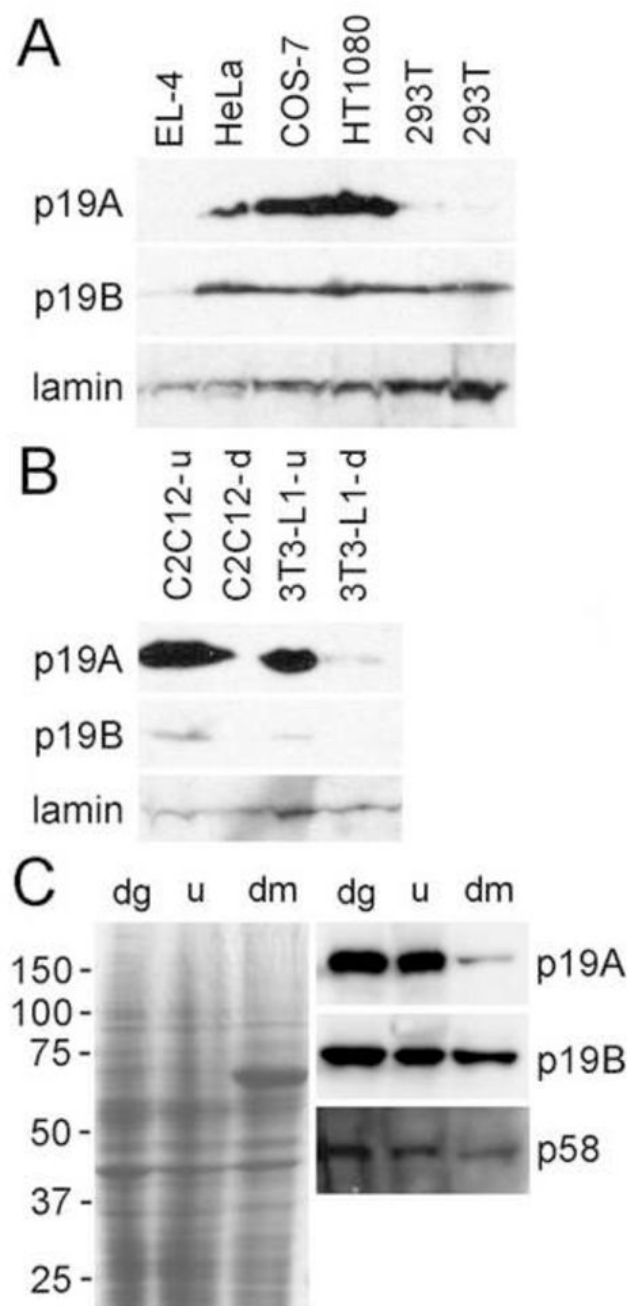
**Fig. 2.**

p19A and p19B have different subcellular localizations. (A) Equal concentrations of rat liver NEs (NE) and microsomes (MM) were probed with antibodies to p19A (a, b) and p19B (c, d). Both p19A antibodies reacted only with NEs while both p19B antibodies reacted only with microsomes. (B) NEs extracted with 500 mM NaCl or 0.1 N NaOH. After salt or alkali treatment insoluble material was pelleted and an equal percentage of supernatant (s) and pellet (p) was analyzed by Western blot with LAP2 β and p19A antibodies. Both LAP2 β and p19A resisted salt extraction, remaining fully in the pellet. In contrast, almost no LAP2 β was extracted with alkali treatment while most p19A was extracted.

**Fig. 3.**

Protein levels of p19A are variable in primary human myoblasts and elevated levels cannot be clearly correlated with FSHD. (A) Myoblast lines from four FSHD patients (p) and controls (c) were tested for levels of p19A. In general, levels were increased in patients, but the correlation was not absolute. (B) Antibodies to p19A (left) failed to recognize the protein in adult muscle samples (lanes 1–5) from FSHD patients and controls, though they recognized the protein in a HeLa extract (h) on the same gel. The same samples were run on a separate gel and reacted with antibodies to A/C lamins (right), confirming sufficient protein was loaded in the lanes to detect p19A if present. Lane 1 clinically diagnosed with Biliary cirrhosis and muscle biopsy “Nearly normal.” Lane 2 clinically diagnosed with Progressive supra-nuclear

palsy and muscle biopsy “Normal.” Lanes 3 and 4 both clinically diagnosed with FSHD and muscle biopsy “Mild myopathy.” Lane 5 clinically diagnosed with Mononeuritis multiplex and muscle biopsy “Nearly normal.” (C) Myoblast lines from unaffected human donor (c) and patients with FSHD (p) were cultured and differentiated *in vitro*. In both myoblasts from controls and patients with FSHD, though the initial levels of p19A varied, the protein levels were reduced during myoblast (u) to myotube (d) differentiation. h, HeLa cell extract.

**Fig. 4.**

p19A disappears during differentiation in certain *in vitro* systems. (A) Levels of p19A are lower in more rapidly dividing cell lines. Protein lysates from various cell lines were loaded on gels after levels were equilibrated with Coomassie staining and reacted with antibodies to p19A, p19B, or lamin B1. Cell doubling times were measured from the same cultures prior to making lysates. EL-4 and 293T cells doubled in roughly half the time of the other cells and p19A levels were also much lower. (B) Levels of p19A were reduced during C2C12 and 3T3-L1 differentiation. Protein levels were equilibrated by Coomassie staining. Levels of p19A were reduced to undetectable levels in the differentiated C2C12 cells and to very low levels in the differentiated 3T3-L1 cells. Levels of p19B were also reduced, unlike for human primary

myoblast differentiation. Levels of lamin B1 were relatively unaffected by the differentiation. (C) Levels of p19A and p19B during HL-60 differentiation. HL-60 cells were differentiated into granulocyte-like cells (dg) or macrophage-like cells (dm) or left undifferentiated (u). An equivalently loaded Coomassie blue stained gel shows that similar amounts of total protein are being compared for all conditions (left). Blots were reacted with antibodies to p19A, p19B and the nucleoporin p58 as a control (right). p19A levels were reduced when cells were differentiated into megakaryocytes, but not when they were differentiated into granulocytes.